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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Overexpression of the Her2 protein has been found in ~30% of breast tumors, and the inhibition of Her2 expression may be an effective way to treat Her2-positive patients. I proposed high-throughput screen of chemical libraries to isolate small molecules that inhibit Her2 transcription by disrupting the interaction of two cancer-linked nuclear proteins, ESX and Sur-2(DRIP130). The compound that we named adamanolol was discovered from a chemical library of 2,422 organic compounds that are all equipped with an indole-like electron-rich pharmacore. The drug-like compound selectively impaired the viability of Her2 positive breast cancer cell lines and reduced the expression of Her2 protein in Her2 positive SK-BR3 cells. In addition, adamanolol competed with FITC-labeled $ESX_{129-145}$ for the interaction with Sur-2 *in vitro*. These results support hypothesis that Sur-2 is a chemically tractable protein that plays an important role in overexpressing Her2 in breast tumors and may serve as a pharmaceutical target for cancer therapy.

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Introduction

Overexpression of the Her2 protein has been found in ~ 30% of breast tumors[1]. Despite the recent success of Her2 receptor-targeted therapeutics, emerging resistance mechanisms now point to the clinical need for additional therapeutic alternatives and combinatorial approaches. In theory, down-regulation of Her2 may be accomplished efficiently by inhibiting the expression of the Her2 gene rather than targeting elevated levels of the Her2 proteins that are already overexpressed. Thus, discovering a means to provide external control over Her2 expression, particularly through small organic molecules, remains appealing. The purpose of this study in the first year is to isolate small molecules that inhibit Her2 transcription.

Body

Preliminary results indicate that the protein levels of Her2 can be decreased by inhibiting the activity of ESX, an epithelial-restricted transcription factor that strongly activates the transcription of the Her2 gene in breast cancer cells. As a first step to isolate small molecules that inhibit Her2 transcription, the 2,422 indole-like compounds were individually assayed at multiple concentrations for their ability to impair the viability of Her2-overexpressing SK-BR3 cells. Among the selected compounds, the one that we named adamanolol impaired the ability of the ESX activation domain to stimulate transcription in cells, whereas it had little effects on those of the activation domains of VP16 and NF-κB (Fig 2A in the appended manuscript), two functionally irrelevant but structurally similar activation domains to the ESX activation domain[2, 3]. Adamanolol impaired the viability of Her-2 positive breast cancer cell lines (SK-BR3, MDA-MB453, and MCF-7), but had much milder effects on MDA-MB468 with no detectable levels of Her2 (Fig 2B in the appended manuscript). Western blot analyses of drug-treated cells showed that the expression of Her2 protein but not that of α-tubulin was significantly reduced by adamanolol in the Her2-positive SK-BR3 cells (Fig 2C in the appended manuscript). Although the expression of other proteins may be influenced by adamanolol, the impaired expression of Her2 is likely to be an important driving force for the selective cell death in Her2-positive cells. The cellular effects of adamanolol are strikingly similar to those observed when an α-helical peptide inhibitor derived from ESX ($ESX_{129\text{-}145}$) was introduced into cells, implicating that adamanolol directly inhibits the ESX-Sur2 interaction[4]. To demonstrated that, I examined if adamanolol competes with fluorescein isothiocyanate (FITC)-labeled $ESX_{129\text{-}145}$ for the interaction with Sur2 *in vitro*. Adamanolol inhibited the ESX-Sur2

interaction in a dose-dependent manner (Fig 2D in the appended manuscript). The interaction was severely impaired at an IC_{50} of ~8 μM, consistent with the results of the cell-based assays. These results support the noton that adamanolol exerts its biological activity by directly disrupting the ESX-Sur2 interaction.

Key research accomplishments

1. Isolation of a small molecule (adamanolol) that inhibits Her2 transcription.
2. Evaluation of adamanolol in cell culture and *in vitro*.

Reportable outcomes

1. Asada, S., **Choi, Y.**, Yamada, M., Wang, S., Hung, M., Qin, J., and Uesugi, M. (2002) External control of Her2 expression and cancer cell growth by targeting a ras-linked coactivator. *Proc. Natl. Acad. Sci USA* 99, 12747-12752

2. Asada, S., **Choi, Y.**, and Uesugi, M. (in press) A gene-expression inhibitor that targets an α-helix-mediated protein interaction. *J. Am. Chem. Soc.*

Conclusions

The small molecule that we named adamanolol was discovered by cell-based screening of chemical libraries. The drug-like molecule showed strong cytotoxicity against Her2-overexpressing breast cancer cells and downregulated expression of Her2 in SK-BR3 cells. Overexpression of Her2 is associated with poor overall survival and enhances malignancy and the metastatic phenotypes in breast cancers[5-9]. Adamanolol could serve as drug lead for breast cancer therapy. Future studies will be focused on evaluation of adamanolol and its derivatives in animal models.

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Appendix: attached

A gene-expression inhibitor that targets an α-helix-mediated protein interaction

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Protein-protein interactions are harder to target by small organic molecules than enzymes or nuclear hormone receptors. Protein-protein binding typically occurs over a relatively large surface area, and the binding surfaces between two proteins tend to be flat and often lack in pockets that might provide binding sites suited for small organic molecules. Nevertheless, protein-protein interfaces vary widely in nature from one to another, and some are likely to present better druggability than others.[1] A good example is the interaction between the somatostatin receptor and β-turn peptide ligands. The success of β-turn-mimicking molecules provided a basis for the notion that the protein-protein interactions mediated by a β-turn peptide are generally more druggable than others.[2]

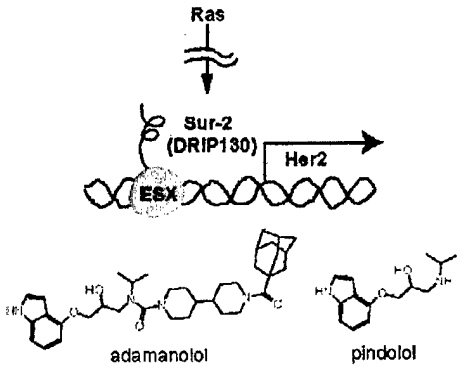

Figure 1. The interaction of the activation domain of ESX with the Ras-linked coactivator Sur-2/DRIP130 is required for the overexpression of the *Her2* oncogene. We found adamanolol, a derivative of pindolol, as an inhibitor of the interaction.

Recent studies suggest that the protein-protein interactions that are mediated by short α-helical segments of proteins are similarly tractable to inhibition by small nonpeptidic molecules.[3] Helical peptide segments of proteins are responsible for a number of biologically important protein associations in the fields of signal transduction and gene transcription. For our case study, we focused on the interaction between the two cancer-linked nuclear proteins, ESX (an epithelial-specific transcription factor) and Sur-2/DRIP130 (a Ras-linked subunit of the human mediator complex).[4] This recently identified interaction is important for the overexpression of the *Her2* oncogene in malignant breast cancer cells (**Fig. 1A**)[4] and thus serves as a potential therapeutic target for Her2-positive breast cancers amounting to 60,000 cases per year in the United States.

The interaction is mediated by one face of an eight-amino-acid α-helical region in the transcriptional activation domain of ESX (Ser-**Trp**-Ile-Ile-Glu-Leu-Leu-Glu), and the tryptophan residue in the hydrophobic face of the helix makes a unique contribution to the specificity of the interaction.[4] The relatively small size of the interface and the importance of the tryptophan residue suggested

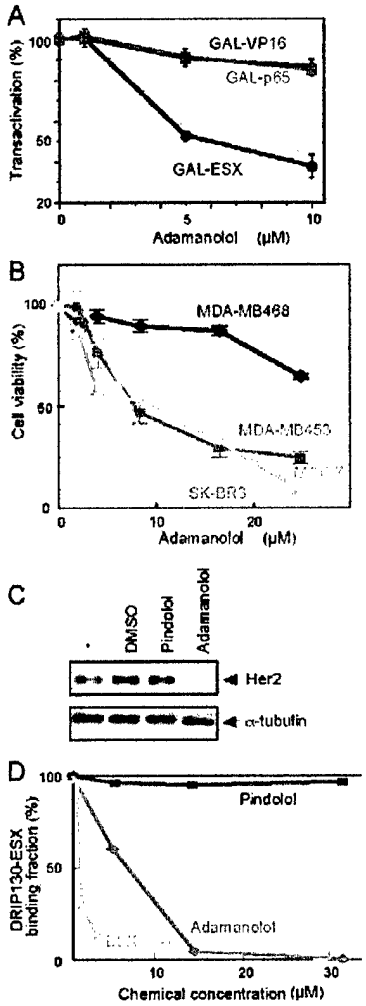

Figure 2. Biological activity of adamanolol. (A) Adamanolol selectively inhibits the ability of the ESX activation domain to activate transcription of secreted alkaline phosphatase reporter gene in HEK293T cells. (B) Adamanolol selectively impairs the viability of Her2-positive breast cancer cell lines (MDA-MB453, MCF-7, and SK-BR3) but has milder effects on MDA-MB468 with no detectable levels of Her2. Cells were treated by adamanolol for 24 hours, and cell viability was estimated by WST metabolic assay. (C) Inhibition of Her2 protein expression by adamanolol. SK-BR3 cells were treated by adamanolol (7.3 μM) for 24 hours, and cell lysates were analyzed by western blots. (D) Adamanolol competes with fluorescein isothiocyanate (FITC)-labeled $ESX_{129\text{-}145}$ (10 nM) for the interaction with Sur-2 (50 μM) in vitro. Pindolol, a truncated derivative of adamanolol, had no effects even at 30 μM.

the existence of small-molecule inhibitors in a chemical library enriched in the structural families of indole, benzimidazole, and benzodiazepin — indole-mimicking π-electron-rich pharmacores

found in bioavailable drugs. We screened 2,422 indole-like compounds by cell-based assays and found the compound that we named adamanolol (**Fig. 1B**).[5] The drug-like pindolol derivative impaired the ability of the ESX activation domain to stimulate transcription in cells, whereas it had little effects on those of the activation domains of VP16 and NF-κB p65, two functionally irrelevant but structurally similar activation domains to the ESX activation domain (**Fig. 2A**).[6] Adamanolol impaired the viability of Her2-positive breast cancer cell lines (SK-BR3, MDA-MB453, and MCF-7), but had much milder effects on MDA-MB468 with no detectable levels of Her2 (**Fig. 2B**).[4] Western blot analyses of drug-treated cells showed that the expression of Her2 protein, but not that of α-tubulin, was significantly reduced by adamanolol in the Her2-positive SK-BR3 cells (**Fig. 2C**). Although the expression of other proteins may be influenced by adamanolol, the impaired expression of Her2 is likely to be an important driving force for the selective cell death in Her2-positive cells.

The cellular effects of adamanolol are strikingly similar to those observed when an α-helical peptide inhibitor derived from ESX ($ESX_{129-145}$) was introduced into cells,[4] implicating that adamanolol directly inhibits the ESX-Sur-2 interaction. To demonstrate that, we examined if adamanolol competes with fluorescein isothiocyanate (FITC)-labeled $ESX_{129-145}$ for the interaction with Sur-2 in vitro. As shown in **Fig. 2D**, adamanolol inhibited the ESX-Sur-2 interaction in a dose-dependent manner.[7] The interaction was severely impaired at an IC_{50} of ~8 μM, consistent with the results of the cell-based assays. These results support the notion that adamanolol exerts its biological activity by directly disrupting the ESX-Sur-2 interaction.

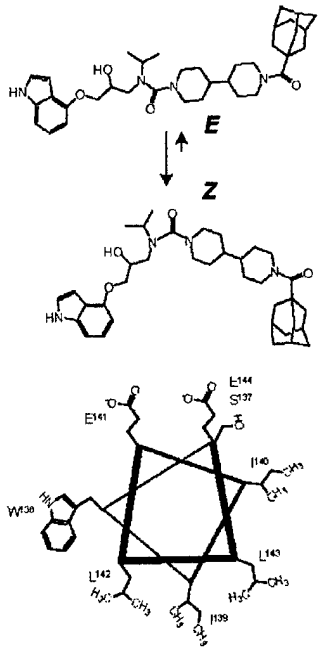

Figure 3. Two possible conformers of adamanolol. NMR analysis of adamanolol suggested its preference of the *Z* conformer imposed by the bulky isopropyl group. The *Z* conformation may play a role in forming a helix-like interface as indicated in the helical wheel presentation of the natural ligand, $ESX_{137-144}$.

It remains unclear how adamanolol inhibits the α-helix mediated interaction. Two close analogs of adamanolol, in which the adamantane group was replaced by a thiophene or toluene group, had little biological activity, suggesting the importance of the bulky, hydrophobic adamantane group. NMR analyses of adamanolol showed signal broadening of the protons around the urea linker at a room temperature and the absence of NOEs between the isopropyl protons and the piperidine even with a 500 msec NOESY mixing time.[8] A reasonable explanation for these NMR characters is a rigid conformation around the urea linker with the preference for the *Z* conformer imposed by the bulky isopropyl group (**Fig. 3**). The *Z* conformation perhaps plays a role in bringing the adamantan group and the indole ring into proximity and forming a helix-like surface for the interaction (**Fig. 3**).[9]

Complete analysis of human genome is anticipated to identify an unprecedented number of potential drug targets. Among these genomic pseudo-targets, the "relatively easy" targets such as nuclear hormone receptors or enzymes will certainly be an immediate focus in pharmaceutical industries. However, more challenging genomic targets including protein-protein interactions in transcriptional regulation need to be assessed to extend the scope of druggable targets for the future drug discovery. Continued efforts targeting the ESX-Sur-2 interaction may serve as a unique case study.

Acknowledgment We thank S. Wang and M. Hung for supplying breast cancer cell lines, Y. Xia, Y. Kwon, and X. Gao for assistance in NMR experiments, H. F. Gilbert for an access to his fluorescence spectrometer, and the members of the Uesugi laboratory for encouragement and discussion. NMR spectra were obtained from the Keck NMR facility at the University of Houston. This work was supported in part by grants from Concern Foundation and Welch Foundation. Y. C. is a predoctoral fellow of US Department of Army.

Supporting Information Available: Details of the experimental procedures and Figures S1 and S2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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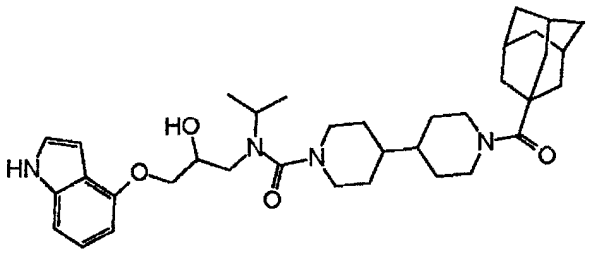
(5) Poor cell-permeability is often a problem of inhibitors of protein-protein interactions. In our study, we performed cell-based screens to eliminate non-cell-permeable molecules. Details of the cell-based screens are shown in Supporting Information.
(6) The activation domains of VP16 and NF-κB p65 served as an excellent control because both have been reported to be α helical [Uesugi, M.; Nyanguile, O.; Lu, H.; Levine, A. J.; Verdine, G. L. *Science* **1997**, *277*, 1310-1313; Schmitz, M. L.; Silva, M. A.; Altman, H.; Czisch, M.; Holak, T. A.; Baeuerle, P. A. *J. Biol. Chem.* **1994**, *269*, 25613-25620.] and are as potent as that of ESX in cells. However, it is possible that adamanolol is not completely specific for the ESX activation domain.
(7) The estimated dissociation constant of FITC-labeled $ESX_{129-145}$ for Sur-2 was 12 μM under the same condition (Fig. S1).
(8) Complete assignments of the proton signals of adamanolol were achieved through TOCSY, DQF-COSY, and NOESY experiments. A summary of key NOE connectivities is shown in Fig. S2.
(9) A less constrained adamanol derivative that lacks the isopropyl group had no detectable activity in SK-BR3 cells even at 100 μM, supporting the notion that the *Z* conformation is important for the activity (Uesugi, M., Shimogawa, H., Kigoshi, H., unpublished results).



ABSTRACT Protein-protein interactions are harder to target by small organic molecules than enzymes or nuclear hormone receptors. Here we report the discovery of an organic compound that inhibits the expression of *Her2* oncogene by disrupting an α-helix-mediated protein interaction. The drug-like molecule we named adamanolol competitively inhibited the interaction between the two cancer-linked nuclear proteins, ESX (an epithelial-specific transcription factor) and Sur-2/DRIP130 (a Ras-linked subunit of the human mediator complex), which is important for the overexpression of *Her2* gene in malignant breast cancer cells. Adamanolol impaired Her2 expression and caused cell death selectively in Her2-positive breast cancer cells. NMR signals of adamanolol suggest that its rigid conformation plays a role in forming a helix-like surface for the interaction.